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(54) Title: IMMUNIZING AGAINST HIV INFECTION

(57) Abstract: A virus neutralizing level of antibodies to a primary HIV isolate is generated in a host by a prime-boost administration of antigents. The primary antigen is a DNA molecule encoding an envelop glycoprotein of a primary isolate of HIV-1 while the boosting antigen is either a non-infectious, non-replicating HIV-like particle having the envelope glycoprotein of a primary isolate of HIV-1 or an attenuated viral vector expressing an envelope glycoprotein of a primary isolate of HIV-1.

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TITLE OF INVENTION

IMMUNIZING AGAINST HIV INFECTION

FIELD OF THE INVENTION

[0001] The present invention relates to the field of immunology and, in particular, to methods and compositions for immunizing a host against infection with HIV.

BACKGROUND OF THE INVENTION

[0002] Human immunodeficiency virus is a human retrovirus and is the etiological agent of acquired immunodeficiency syndrome (AIDS). It is estimated that more than 33 million people have been infected with HIV world-wide as of December 1999 (Ref 1- various references are referred to in parenthesis to more fully describe the state of the art to which this invention pertains. Full bibliographic information for each citation is found at the end of the specification, immediately preceding the claims. The disclosure of these references are hereby incorporated by reference into the present disclosure).

[0003] As the HIV epidemic continues to spread world wide, the need for an effective vaccine remains urgent. Efforts to develop such a vaccine have been hampered by several factors three of which are: (a) the extraordinary ability of the virus to mutate; (b) inability of most known specificities of anti-HIV antibodies to neutralise HIV primary isolates consistently; and (c) lack of understanding of the correlates of protective immunity to HIV infection. Over the last 10 years, several candidate HIV vaccines have been tested in primates for their immunoprotective abilities (Ref. 2). These studies suggest that both neutralising antibodies and cell-mediated immunity play a role in conferring sterilizing immunity and preventing progression towards disease (Ref 3, 4). While the correlates for immune protection against HIV-1 infection are currently unknown, an effective HIV vaccine should elicit both strong neutralising antibody and cytotoxic T lymphocyte (CTL) responses.

[0004] Envelope subunit vaccines have been shown to induce high titred humoral responses, but were inefficient in eliciting CTL responses (Ref 5). Live recombinant pox vectors have been shown to elicit very potent CTL responses, however these vectors were ineffective for generating a significant antibody

response (Ref 6). In attempts to combine the two immunization types, several clinical trials involved a prime-boost strategy, consisting of initial viral vector immunization followed by boosts with recombinant HIV-1 envelope subunits (Ref 7, 8), have led to limited success with respect to CTL responses. Other vaccine approaches have used non-infectious, non-replicating, immunogenic virus-like particles (VLP) for immunising against HIV infection (Ref 9, 10). This type of immunogen has lead to the generation of neutralizing antibodies to a laboratory HIV-1 strain (Ref 10).

[0005] A prime-boost approach has been investigated using non-infectious VLPs to enhance HIV-specific CTL responses in mice primed with recombinant canarypox vector vCP205 encoding HIV-1gp 120 (MN strain) (Ref 11). This study showed that VLPs could boost the CTL response to the canarypox vector.

[0006] Recently, a study showing the induction of neutralizing antibodies to a HIV-1 primary isolate in chimpanzees has been reported (Ref 12). In this study, recombinant adenovirus expressing gp160 was used as the priming agent and recombinant gp120 protein was used to boost the monkeys.

[0007] There is still a need for vaccines and immunization regimes to induce both a strong CTL response as well as neutralizing antibodies to HIV primary isolates.

SUMMARY OF THE INVENTION

[0008] In accordance with one aspect of the present invention, there is provided a method for generating, in a host, particularly a human host, a virus neutralizing level of antibodies to a primary HIV isolate, comprising at least one administration of a priming antigen to the host, wherein the priming antigen comprises a DNA molecule encoding an envelope glycoprotein of a primary isolate of HIV, resting the host for at least one specific resting period to provide for clonal expansion of an HIV antigen specific population of precursor B-cells therein to provide a primed host, and at least one administration of a boosting antigen to the primed host to provide said neutralizing levels of antibodies, wherein the boosting antigen is selected from the group consisting of a non-infectious, non-replicating, immunogenic HIV-like particle having at least part of the envelope glycoprotein of a primary isolate of HIV and an attenuated viral

vector expressing at least part of an envelope glycoprotein of a primary isolate of HIV.

[0009] The primary HIV isolate may be an HIV-1 isolate including from the clade B HIV-1 clinical isolate HIV-1_{Bx08}, although any other primary HIV-1 isolate may be employed in the immunization procedures of the invention.

[0010] The DNA molecule encoding the envelope glycoprotein of a primary isolate of HIV may be contained in a plasmid vector under the control of a heterologous promoter, preferably a cytomegalovirus promoter, for expression of the envelope glycoprotein in the host, which may be a human host.

[0011] The vector utilized for DNA molecule immunization is novel and constitutes a further aspect of the present invention. Preferably, the vector has the identifying characteristics of pCMV3Bx08 shown in Figure 2, such identifying characteristics being the nucleic acid segments and restriction sites identified in Figure 2.

[0012] A priming administration of antigen may be effected in a single or in multiple administrations of the priming antigen. In the latter case, the at least one specific resting period to permit clonal expression of HIV antigen-specific population precursor B-cells may be effected after each priming administration. The at least one specific resting period may be between about 2 and 12 about months.

[0013] In the embodiment where the boosting antigen is a non-infectious, non-replicating, immunogenic HIV-like particle, such particle may comprise an assembly of:

(i) an *env* gene product,

(ii) a *pol* gene product, and

(iii) a *gag* gene product

with the particle being encoded by a modified HIV genome deficient in long terminal repeats (LTRs) and containing gag, pol and env in their natural genomic arrangement. Such particles and the manufacture thereof are described in US Patent No. 5,439,809, assigned to the assignee hereof and the disclosure of which is incorporated herein by reference. Such particles can include mutations in gag and pol to further reduce potential infectivity, as more fully described in United

States Patent No. 6,080,408, assigned to the assignee hereof and the disclosure of which is incorporated herein by reference (WO 96/06177). In a preferred embodiment, the *env* gene is that from primary isolate BX08. The *gag* gene and *pol* gene may be those from the same primary isolate or may be chosen from those of other HIV-1 isolates, which may be primary isolates.

[0014] The non-infectious, non-replicating, immunogenic HIV-like particle may be administered in conjunction with an adjuvant. Any suitable adjuvant may be used, such as QS21, DC-chol, RIBI or Alum.

[0015] Such non-infectious, non-replicating, immunogenic HIV particle may be formed by expression from a suitable vector in mammalian cells. In accordance with an additional aspect of this invention, there is provided a vector comprising a modified HIV-genome deficient in long terminal repeats and a heterologous promoter operatively connected to said genome for expression of said genome in mammalian cells to produce the non-infectious, non-replicating and immunogenic particle, wherein at least the *env* gene of the modified HIV-genome is that from a primary isolate of HIV. The *gag* and *pol* genes of the modified HIV genome may be those from the same primary isolate or those from another isolate, which may be a primary isolate.

[0016] The vector preferably is a plasmid vector while the primary isolate preferably is BX08. The promoter may be the metallothionein promoter. The vector preferably has the identifying characteristics of plasmid p133B1 shown in Figure 3, such identifying characteristics being the nucleotide segments and restriction sites identified in Figure 3.

[0017] In the embodiment where the boosting antigen is an attenuated viral vector, the attenuated viral vector may be an attenuated avipox virus vector, particularly the attenuated canary poxvirus ALVAC. The attenuated viral vectors used herein form another aspect of the invention. The attenuated viral vector may contain a modified HIV genome deficient in long terminal repeats (LTRs), wherein at least the *env* gene is that from primary isolate BX08. The *gag* and *pol* genes of the modified genome may be those from the same primary isolate or may be chosen from other HIV isolate.

[0018] The attenuated canarypox virus-based vector ALVAC is a plaque-cloned derivative of the licensed canarypox vaccine, Kanapox, and is described in reference 19. The attenuated canary pox vector preferably has the identifying characteristics of vCP1579 shown in Figure 4, such identifying characteristics being the nucleic acid segments and restriction sites identified in Figure 4.

[0019] The at least one administration of a boosting antigen may be effected in a single administration or at least two administration of the boosting antigen.

[0020] The invention further includes compositions comprising the immunogens as provided herein and their use in the manufacture and formulation of immunogenic compositions including vaccines.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] The present invention will be further understood from the following description with reference to the drawings, in which:

Figure 1 shows the details of the elements of plasmid pCMVgDtat⁻vpr⁻Bx08.

Figure 2 shows the details of the elements of plasmid pCMV3Bx08.

Figure 3 shows the details of the elements of plasmid p133B1.

Figure 4 shows the details of the insertions into ALVAC (2) to provide vector vCP1579.

Figures 5A and 5B contain a representation in time-line form of the immunization regime used wherein the study groups are described in Table 1. The numbers below the lines refer to weeks.

Figure 6 shows the immunoreactivity to HIV-1 antigens of the serum diluted 1:100 from the macaques immunized with the various preparations as described in Table 1.

Figure 7 shows the immunoreactivity to HIV-1 antigens of the serum diluted 1:1000 from the macaques immunized with the various preparations as described in Table 1.

Figure 8 shows the details of the elements of pMPC6H6K3E3.

Figure 9 shows the details of the elements of pMPC5H6PN.

Figure 10 shows the details of the elements of pHIV76.

Figure 11 shows the nucleotide sequence (SEQ ID NO: 1) for the H6/HIV Pol/Nef epitope cassette in the ALVAC C5 site of vCP1579.

Figure 12 contains the nucleotide sequence of C6 region (coding strand SEQ ID NO: 16, complementary strand SEQ ID NO: 17, K3L amino acid sequence SEQ ID NO: 18, E3L amino acid sequence SEQ ID NO: 19).

GENERAL DESCRIPTION OF INVENTION

[0022] As noted earlier, the present invention involves administration of HIV antigens to elicit virus-neutralizing levels of antibodies against a primary HIV isolate.

[0023] A DNA construct was prepared incorporating the *env* gene from the primary isolate Bx08 under the control of the cytomegalovirus promoter and the construct, pCMV3Bx08, is shown in Figure 2. The construct pCMV3Bx08 is derived from plasmid pCMVgDtat$^-$vpr$^-$Bx08 seen in Figure 1. The DNA construct pCMV3Bx08 was used in a priming immunization step to a host, macaque monkeys being the animal model chosen.

[0024] Following the priming immunization step, which may be effected in one or more administrations of the DNA construct, the host is allowed to rest to provide for clonal expression of an HIV antigen specific population of precursor B-cells therein to provide a primed host.

[0025] The boosting administration is effected either with a non-infectious, non-replicating, immunogenic HIV-like particle (VLP) or an attenuated viral vector.

[0026] For this purpose, a VLP expression plasmid was constructed containing a modified HIV genome lacking long terminal repeats in which the *env* gene is derived from primary isolate BX08, wherein the modified HIV genome is under the control of a metallothionein promoter. The construct, p133B1, shown in Figure 3, was used to effect expression in mammalian cells of the non-infectious, non-replicating, immunogenic HIV-like particules, in which the *env* gene product is that from the primary isolate BX08.

[0027] In the case of the attenuated virus vector, a recombinant attenuated canarypox virus vector was constructed to contain the *env* gene from primary

isolate BX08. The viral vector vCP1579 (Figure 4) was prepared by a variety of manipulatious from plasmid pHIV76 (Figure 10), as shown described in detail below.

[0028] These products were utilized in a boosting administration to the primed macaques. The boosting administration may be effected in one or more immunizations. In a preferred aspect of the invention, the non-infectious, non-replicating immunogenic HIV-like particles may co-administered with the DNA construct in the priming administration and the DNA construct may be coadministered with the HIV-like particles in the boosting administration.

[0029] Immunizations were effected in accordance with the procedure of the invention and the results obtained were compared with those obtained using other protocols according to the protocols set forth in Table 1. The immunization regimes used are shown as time lines in Figures 5A and 5B.

[0030] The results obtained following the various protocols showed that, in particular, a primary DNA vaccination in combination with a boost from either the VLP or the attenuated canarypox virus enhanced the levels of neutralizing antibodies, as indicated by the reduction of detectable p24 levels in cells infected with primary HIV isolates.

Biological Deposits

[0031] Certain vectors that are described and referred to herein have been deposited with the American Type Culture Collection (ATCC) located at 10801 University Boulevard Manassas, Virginia 20110-2209, USA, pursuant the Budapest Treaty and prior to the filing of this application. Samples of the deposited vectors will become available to the public and all restrictions imposed or access to the deposits will be received upon grant of a patent based on this United States patent application or the United States patent application in which they are described. In addition, the deposit will be replaced if viable samples cannot be dispensed by the Depository. The invention described and claimed herein is not limited in scope by the biological materials deposited, since the deposited embodiment is intended only as an illustration of the invention. Any equivalent of similar vectors that contain nucleic acids which encode equivalent or

similar antigens as described in this application are within the scope of the invention.

Deposit Summary

Plasmid	ATCC	Deposit Date
pMT-HIV	40912	October 12, 1990
pCMVgDtat$^-$vpr$^-$	209446	November 11, 1997

EXAMPLES

[0032] The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitation.

[0033] Example 1

[0034] This Example describes the construction of plasmid pCMV3BX08.

[0035] The plasmid, pCMV3BX08, contains sequence segments from various sources and the elements of construction are depicted in Figure 2.

[0036] The prokaryotic vector pBluescript SK (Stratagene) is the backbone of the plasmid pCMV3.BX08 and was modified by the replacement of the *Amp*^{R} with *Kan*^{R} gene and the deletion of the fl and the LacZ region. To achieve the desired modifications, the sequence between *Ahd*I (nucleotide 2,041) and *Sac*I (nucleotide 759) of pBluescript SK, which contains the *Amp*^{R}, fl origin and the LacZ, was deleted. A 1.2 kb *Pst*I fragment from the plasmid pUC-4K (Pharmacia) containing the *Kan*^{R} gene, was blunt end ligated to the *Ahd*I site of pBluescript SK in a counter-clockwise orientation relative to it's transcription. A 1.6 kb *Ssp*I/*Pst*I DNA fragment containing the human cytomegalovirus immediate-early gene promotor, enhancer and intron A sequences (CMV) was ligated to the other end of the *Kan*^{R} gene so that the transcription from the CMV promoter proceeds in the clockwise orientation. A synthetic oligonucleotide segment containing translation initiation sequence and sequences encoding the human tissue plasminogen activator signal peptide (TPA) was used to link the

CMV promotor and the sequences encoding the envelope gene of the primary isolate HIV-1_{BX08}.

[0037] The envelope gene from the HIV-1 primary isolate BX08 was isolated from the plasmid pCMVgDtat$^-$vpr$^-$Bx08 illustrated in Figure 1. The plasmid pCMVgDtat$^-$vpr$^-$Bx08 was derived from the deposited plasmid pCMVgDtat$^-$vpr$^-$, the construction of which is described in copending United States Patent Application No. 08/991,773 filed December 16, 1997, assigned to the assignee hereof and the disclosure of which is incorporated herein by reference, (WO 99/31250). The plasmid pCMVgDtat$^-$vpr$^-$Bx08 was derived by substituting the BX08 envelope sequence from clade B HIV-1 clinical isolate HIV-1_{BX08} for the modified HIV genome sequence present in pCMVgDtat$^-$vpr$^-$. Plasmid pCMVgDtat$^-$vpr$^-$Bx08 was restricted with the restriction enzyme *Xho* I and made blunt ended with Klenow treatment. A *Not* I partial digestion was then performed and the resulting 6.3 kb fragment containing the *env* gene was isolated. Plasmid pCMV3 (Invitrogen) was restricted with *Bam* HI and made blunt ended with Klenow treatment. The plasmid pCMV3 was then restricted with *Not* I and the resulting 4.4 kb fragment was isolated. The 6.3 and 4.4 kb fragments were ligated together to produce plasmid pCMV3BX08 (Figure 2).

[0038] The pCMV3BX08 construct was introduced into HB101 competent cells according to manufacturer's recommendations (GibcoBRL). Correct molecular clones were identified by restriction and sequencing analysis and their expression of envelope glycoprotein was examined in transient transfections followed by Western blot analysis.

[0039] All DNAs used for immunizations were prepared using EndoFree Plasmid Kit (Qiagen). For intramuscular immunizations either 3 mg or 600 µg of pCMVBX08, in 100 µl PBS was injected.

[0040] Proviral DNA for clade B HIV-1 clinical isolate HIV-1_{BX08} originated at Transgene (Strasbourg, France) and was isolated from genomic DNA of cells infected with the virus.

[0041] Example 2

[0042] This Example describes the construction of plasmid p133B1.

[0043] A Bx08 plasmid expression vector (p133B1, Figure 3) used to transfect the mammalian cells was engineered in several stages using pUC18 as the initial host plasmid. First, an 8.3-kbp fragment of HIV-1_{LAI} provirus encoding the gag, pol and env proteins was isolated. This fragment lacked the transcription regulatory elements and long terminal repeat elements from each end of the proviral genome to ensure the virus-like particles would be replication-incompetent. This fragment was linked to an inducible human type IIA metallothionein (MTIIA) promoter (Ref 13) and also to a Simian Virus 40 polyadenylation (polyA) addition/transcription termination sequence from plasmid pSV2dhfr (Ref 14). The modified fragment was then inserted into the pUC18 host vector. The resulting deposites expression construct, named pMT-HIV, was used to transfect into African green monkey kidney (Vero) and COS monkey kidney cells. The procedure for obtaining pMT-HIV is further described in the aforementioned US Patent No. 5,439,809. Both transfected cell lines produced non-replicating virus-like particles when induced with metal ions (Ref 15).

[0044] Two further modifications were made to the proviral DNA in pMT-HIV to provide additional safety features to protect human cells against recombination events with reverse-transcribed DNA:

1) inactivation of the RNA packaging sequences; and
2) deletion of a large section of the *pol* gene encoding reverse transcriptase and integrase.

[0045] To delete the first RNA packaging signal, part of the DNA corresponding to the untranslated leader sequence of the mRNA was replaced with synthetic DNA lacking a 25-bp motif corresponding to nucleotides 753-777 (the *psi* sequence). To inactivate the second RNA packaging signal, two adenosine residues within a *gag* gene zinc finger sequence were changed to thymidine residues. Each of these residue changes had the effect of replacing cysteine residues in a Cys-His array with a serine in the gene product.

[0046] The *pol* gene deletion was effected by replacing a 1.9-kbp fragment with synthetic DNA containing stop codons in all three reading frames. This prevented read-through translation of the residual integrase coding sequence on the 3' side of the deletion. The 1.9-kbp deletion in *pol* also eliminated the

expression of reverse transcriptase and integrase enzymes. However, the deletion left intact the gene encoding the viral protease, which is both an immunogenic component of HIV-1 virus particles and allows the expression of particles with processed gag antigens closely resembling native virions (Ref 16). The protease also contains epitopes that are conserved across HIV-1 clades. The modifications described with respect to *gag* and *pol* genes are more fully described in the aforementioned United States Patent No. 6,080,408 (WO 96/06177).

[0047] Finally, the HIV-1_{LAI} *env* gene within pMT-HIV was replaced with that of HIV-1_{Bx08}. To effect this replacement, a 2440-bp fragment containing the *env* gene of Bx08 was amplified by polymerase chain reaction (PCR) from cells infected with this isolate. The PCR product was then used to replace the corresponding region present in pMT-HIV. However, the incoming fragment from HIV-1_{Bx08} was 125-bp shorter than the original HIV-1_{LAI} region owing to a deletion in the untranslated region between the *env* gene stop codon and the termination/polyA addition sequence. The resulting construct replaced all but eleven amino acid residues of the LAI envelope proteins gp120 and gp41. Of these eleven, only the first three differ between the LAI and Bx08 isolates, and these are all charge-conservative changes meaning the final expression vector (p133B1) encoded a nearly authentic HIV-1_{Bx08} env protein.

[0048] Example 3

[0049] This Example describes the production of HIV-like particles.

[0050] African green monkey kidney (Vero) cells were recovered and cultivated in Dulbecco's modified Eagle medium (DMEM) containing 10% v/v fetal bovine serum (FBS), referred to below as Complete Medium. At passage 141, the cells were transfected with p133B1 using the calcium phosphate method when at approximately 30% confluence. The cells were shocked with glycerol 8 hours after transfection. For this step, six 10-cm dishes containing approximately 3.0×10^6 cells each in 10.0 mL of Complete Medium were prepared. Each dish received 25.0 µg of expression vector and 2.0 µg of plasmid pSV2neo (Ref 17). The pSV2neo contains a selectable marker gene conferring resistance to the antibiotic geneticin (G418). Two days after transfection, the cells from each dish

were recovered by trypsinization and replated into twenty-five fresh dishes in Complete Medium supplemented with 0.5 mg/mL of G418.

[0051] In total, 394 colonies were isolated from the dishes using cloning cylinders. Each colony was recovered by trypsinization and divided into two cluster dish wells, one of the wells per clone was induced after reaching 50% to 90% confluence. Prior to induction, the wells were treated by replacing all the medium with fresh Complete Medium containing 10.0 μM 5-azacytidine. After incubating for between 18 hours and 22 hours, the medium was removed and replaced with fresh DMEM containing 0.2% v/v FBS, 2.0 μM $CdCl_2$ and 200.0 μM $ZnCl_2$. The wells were incubated for a further 20 hours to 24 hours at which time samples of the medium were removed and tested by p24 ELISA.

[0052] The twenty highest-producing clones, based on the p24 titre, were chosen and cells from the corresponding uninduced wells were sub-cultured into one T-25 and one T-150 flask per clone. Both flasks were grown to confluence. The cells from the T-150 were recovered by trypsinization and cryopreserved at passage number 145. The cells from the T-25 were recovered by trypsinization every 3 days to 4 days and maintained up to passage 153. The cells were induced as above and samples retested by p24 ELISA at two different passages prior to passage 153.

[0053] The two highest p24 producers were chosen and were recovered by trypsinization every 3 days to 4 days up to passage 163. Samples from the clones were tested by p24 and gp120 ELISA from passage 158 and by p24 ELISA at passage 163, to assess clonal stability. The most suitable of these two cell lines, named 148 to 391, was chosen for further sub-cloning. The clone nomenclature defines the experiment number for this procedure, which was 148, and the number of the clone, which was number 391 of the original 394 isolated.

[0054] The vero cells were grown for approximately 100 h to 103 h and the medium was then replaced with growth medium containing 5-azacytidine. The bottles were then incubated for a further 20 h to 22 h, at which time the medium was replaced with serum-free medium containing $CdCl_2$ and $ZnCl_2$. The bottles were then incubated for 29 h to 31 h, at which time the medium was harvested, pooled and stored at 2°C to 8°C prior to purification.

[0055] The next day after harvesting, the solution was clarified, concentrated and diafiltered against phosphate buffer. The concentrate was passed through a ceramic hydroxyapatite (type I) column and the run-through was collected. The run-through from two successive sublots was pooled together and pumped onto a sucrose density gradient in a continuous zonal ultracentrifuge rotor. Pseudovirion-containing fractions were collected and pooled. The pooled pseudovirion fractions were diafiltered against PBS containing 2.5% sucrose to reduce the sucrose content, concentrated and diafiltered again. The material was sterile filtered using a 0.2 μm filter. At this stage the materials was designated as a purified sub-lot and were stored at 2 to 8°C.

[0056] The adjuvants were prepared separately and filter sterilized before filling in single dose vials. QS21 was stored at -20°C.

[0057] Example 4

[0058] This Example describes the production of recombinant pox virus vCP1579.

[0059] Recombinant pox virus vCP1579 (Figure 4) contains the HIV-1 gag and protease genes derived from the HIV-1 IIIB isolate, the gp120 envelope sequences derived from the HIV-1 Bx08 isolate, and sequences encoding a polypeptide encompassing the known human CTL epitopes from HIV-1 Nef and Pol.

[0060] Recombinant vCP1579 (Figure 4) was generated by insertion of the vector modifying sequences from pMPC6H6K3E3 (Figure 8) encoding E3L and K3L into the C6 site of recombinant vCP1566 (Figure 4). Recombinant vCP1566 was generated by insertion of an expression cassette encoding a synthetic polypeptide containing Pol CTL epitopes and Nef CTL epitopes (Figure 11) and plasmid pMPC5H6PN (Figure 9) into vCP1453 at the insertion site known as C5. Recombinant vCP1453 was generated by co-insertion of genes encoding HIV-1 env and gag/protease gene products, plasmid pHIV76 (Figure 10), into the ALVAC genome at the insertion site known as C3.

[0061] The construction of recombinant pox vectors containing the E3L and K3L genes has been described in United States patent 6,004,777 issued Dec 21, 1999 to Tartaglia et al. and the recombinant pox vectors describing the

insertion of HIV genes has been described in United States patent 5,766,598 issued June 16 1998 to Paoletti et al.

[0062] The locus designated C3 was used for the insertion of the HIV-1 *env* and *gag* gene sequences into the ALVAC(2) vector, and the locus designated as C5 was the insertion site for the sequences encoding the HIV-1 Nef and Pol CTL epitopes. By virtue of the C3 and C5 loci existing within the extensive inverted terminal repetitions (ITRs) of the virus genome (approximately 41 kbp), insertion into these loci results in the occurrence of two copies of the inserted HIV-1 sequences.

[0063] Briefly, expression cassette pHIV76 (Figure 10) was engineered in the following manner. Plasmid p133B1 (Figure 3) containing the HIV-1Bx08 gp 160 gene was used as the starting plasmid. The 3'-end of the H6 promoter was cloned upstream of the gp160 gene and three poxvirus early transcription termination signal sequences (T_5NT) were modified. This was accomplished by cloning a 2,600 bp *Bam*HI-digested PCR fragment, containing the 3'-end of the H6 promoter and the T_5NT-modified HIV-1 (BX08) gp160 gene, into the *Bam*HI site of pBS-SK. This PCR fragment was generated from four overlapping PCR fragments (a 570 bp fragment, a 140 bp fragment, a 500 bp fragment and a 1,450 bp fragment) and the oligonucleotides, RW835 (5'-ATCATCATCGGATCC CGGGGTCGCGATATCCGTTAAGTTTGTATCGTAATGAAAGTGAAGGAC C-3' - SEQ ID NO: 2) and RW836 (5'-ATCATCATCGGATCCCGGGGTT ATAGCAAAGCCCTTTC-3' - SEQ ID NO: 3). The 570 bp PCR fragment, containing the 3'-end of the H6 promoter and the 5'-end of the gp160 gene, was generated from the plasmid, p133B1, with the oligonucleotides, RW835 (5'-ATC ATCATCGGATCCCGGGGTCGCGATATCCGTTAAGTTTGTATCGTAATG AAAGTGAAGGAGACC-3') and RW868 (5'-ATCAAGACTATAGAAGA GTGCATATTCTCTCTTCATC-3'). The 140 bp PCR fragment, containing an interior portion of the gp160 gene, was generated from plasmid p133-B1 with the oligonucleotides, RW864 (5'-GCACTCTTCTATAGTCTTGATATAGTAC-3' - SEQ ID NO: 4) and RW865 (5'-AGCCGGGGCGCAGAAATGTATG GGAATTGGCAC-3' - SEQ ID NO: 5). The 500 bp PCR fragment, containing an interior portion of the gp160 gene, was generated from 133-3 with the

oligonucleotides, RW866 (5'-ATACATTTCTGCGCCCCGGCTGGT TTTGCGATTC-3' - SEQ ID NO: 6) and RW867 (5'-GAAGAATTC CCCTCCACAATTAAAAC-3' - SEQ ID NO: 7). The 1,450 bp PCR fragment, containing the 3'-end of the gp160 gene, was generated from p133-B1 with the oligonucleotides, RW869 (5'-TGTGGAGGGGAATTCTTCTACTGTAATAC AACACAAC-3' - SEQ ID NO: 8) and RW836 (5'-ATCATCATCGGAT CCCGGGGTTATAGCAAAGCCCTTTC-3' - SEQ ID NO: 9). The 3'-end of the 570 bp PCR fragment overlaps the 5'-end of the 140 bp PCR fragment. The 3'-end of the 140 bp PCR fragment overlaps the 5'-end of the 500 bp PCR fragment. The 3'-end of the 500 bp PCR fragment overlaps the 5'-end of the 1450 bp PCR fragment. The plasmid generated by this manipulation is called pRW997.

[0064] The sequence encoding gp41 was then replaced with the sequence encoding the gp160 transmembrane (TM) region. This modification was accomplished by cloning a 200 bp *Mfe*I-*Hin*dIII-digested PCR fragment, containing the 3'-end of the gp120 gene and the TM sequence, into the 4,400 bp *Mfe*I-*Hin*dIII fragment of pRW997. This PCR fragment was generated from two overlapping PCR fragments (a 170 bp fragment and a 125 bp fragment) with the oligonucleotides, HIVP97 (5'-TAGTGGGAAAGAGATCTTCAGACC-3' - SEQ ID NO: 10) and HIVP101 (5'-TTTTAAGCTTTTATCCCTGCCTAACT CTATTCAC TAT-3' - SEQ ID NO: 11). The 170 bp PCR fragment was generated from pRW997 with the oligonucleotides, HIVP97 (5'-TAGTGGGAAAGAGATCTTCAGACC-3' - SEQ ID NO: 12) and HIVP100 (5'-CCTCCTACTATCATTATGAATATTCTTTTTTCTCTCTGCACCACTCT-3' - SEQ ID NO: 13). The 125 bp PCR fragment was generated from pRW997 with the oligonucleotides, HIVP99 (5'-AGAGTGGTGCAGAGAGAAAAA AGAATATTCATAATGATAGTAGGAGGC-3' - SEQ ID NO: 14) and HIVP101 (5'-TTTTAAGCTTTTA TCCCTGCCTAACTCTATTCACTAT-3' - SEQ ID NO: 15). The plasmid generated by this manipulation is called pHIV71.

[0065] The H6-promoted gp120+TM gene was then cloned between C3 flanking arms, into a plasmid containing the I3L-promoted HIV1 gag/(pro) gene. This modification was accomplished by cloning the 1,600 bp *Nru*I-*Xho*I fragment of pHIV71, containing the H6-promoted gp120+TM gene, into the 8,200 bp *Nru*I-

*Xho*I fragment of pHIV63 . The plasmid generated by this manipulation is called pHIV76 (Figure 10). Plasmid pHIV76 was used in *in vivo* recombination experiments with ALVAC (CPpp) as rescue virus to yield vCP1453.

[0066] The sequence of the nef/pol regions is shown in Figure 12 and the E3L and K3L sequences are shown in Figure 13. To generate ALVAC(2)120(BX08)GNP (vCP1579), expression cassettes consisting of the promoter/HIV-1 gene combinations were subcloned into an ALVAC donor plasmid, which were then used to insert the expression cassettes into defined sites in the ALVAC genome by *in vitro* recombination as previously described (Ref 20).

[0067] Example 5

[0068] This Example describes the results of immunization regimes.

[0069] Groups of four animals (macaques) each were randomly assigned to seven vaccine groups as illustrated in Table 1. In this Table, "BX08 DNA" refers to pCMV3BX08, prepared as described in Example 1, "BX08 VLP" refers to the pseudovirions produced by expression vector p133B1 in Vero cells, as described in Example 3, and "ALVAC(2) BX08" refers vCP1579, prepared as described in Example 4. Reference (pre-bleed) sera were sampled at –6 and –2 weeks pre-vaccination. Primary immunizations with the various vaccines were given on weeks 0 and 4 with boosts on weeks 24 and 44 (Figures 5A, 5B). The vaccines were immunized intramuscularly into one quadricep of each macaque monkey.

[0070] Sera were prepared from whole-blood using SST collection tubes and analyzed using commercially available HIV-1 western blots. Groups 1, 2 and 7 showed low levels of anti-Env antibodies after the first boost (Figures 6 and 7). Based on ELISA values, the anti-env antibody levels were below 1 μg/ml of specific IgG. High levels of anti-gag antibodies were detected in groups 1, 2, 3, 4, and 7 (Figures 6 and 7). No HIV-1 specific antibodies were detected in groups 5 and 6 (Figure 6).

[0071] The ability of the antibodies raised in the immunized monkeys to neutralize HIV-1BX08 virus in human PBMC was assayed based on the reduction of p24 levels.

[0072] The neutralization assay was performed essentially as described in reference 18. Briefly, serum dilutions were mixed with HIV-1 BX08 and the mixtures incubated for 1 hour, then added to susceptible human PBMC cells. Titres were recorded as the dilution of serum at which p24 was reduced by 80%. Serum samples were assayed at 1:2, 1:8 and 1:32 dilution on the virus (1:6, 1:24 and 1:26 dilutions after the addition of cells). p24 levels were evaluated by p24-specific ELISA assay.

[0073] DNA vaccination on its own, group 5, and ALVAC on its own, group 6, had no monkeys showing reduction of p24 levels greater than 80%. The low DNA (600 ug) plus ALVAC, group 4, also showed no monkeys with greater than 80% reduction of p24 titres. VLP plus DNA, either high or low dose (group 1 and 2) showed enhanced reduction of p24 levels compared to VLPs alone, group 7. High dose DNA, group 3, in combination with ALVAC enhanced the ability to elicit p24 or virus neutralising antibodies over the low dose, group 4 or ALVAC alone, group 6. These results indicate that DNA vaccination in combination with VLPs or ALVAC enhanced the levels of virus neutralising antibodies as indicated by the reduction of p24 levels in the sera of the immunized monkeys.

[0074] The percentage reduction of p24 is calculated relative to the amount of p24 produced in the presence of the corresponding dilution of week 2 samples.

SUMMARY OF DISCLOSURE

[0075] In summary of this disclosure, the present invention provides novel immunization procedures and immunogenic compositions for generating virus neutralizing levels of antibodies to a primary HIV isolate and vectors utilized therein and for the generation of components for use therein. Modifications are possible within the scope of this invention.

Table 1 Study Design

Group number	Treatment – Week 0, 4	Treatment – Week 24,44
1	3 mg BX08 DNA 50 µg BX08 VLP 100 µg QS21	3 mg BX08 DNA 50 µg BX08 VLP 100 µg QS21
2	600 µg BX08 DNA 50 µg BX08 VLP 100 µg QS21	600 µg BX08 DNA 50 µg BX08 VLP 100 µg QS21
3	3 mg BX08 DNA	ALVAC(2) BX08 ($1x10^8$ pfu)
4	600 µg BX08 DNA	ALVAC(2) BX08 ($1x10^8$ pfu)
5	3 mg BX08 DNA	3 mg BX08 DNA
6	Control DNA	ALVAC(2) BX08 ($1x10^8$ pfu)
7	50 µg BX08 VLP 100 µg QS21	50 µg BX08 VLP 100 µg QS21

Table 2 Number of Monkeys showing > 80% reduction of p24 titre.

Group number	Week 26 Bleed	Week 44 Bleed
1	3/4	¾
2	3/4	4/4
3	2/4	2/4
4	0/4	0/4
5	0/4	0/4
6	0/4	0/4
7	2/4	3/4

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CLAIMS

What we claim is:

1. A method for generating in a host a virus neutralizing level of antibodies to a primary HIV isolate, comprising:

at least one administration of a priming antigen to the host, wherein the priming antigen comprises a DNA molecule encoding an envelope glycoprotein of a primary isolate of HIV-1,

resting the host for at least one specific resting period to provide for clonal expansion of an HIV antigen specific population of precursor B-cells therein to provide a primed host, and

at least one administration of a boosting antigen to the primed host to provide said neutralizing levels of antibodies, wherein the boosting antigen is selected from the group consisting of a non-infectious, non-replicating, immunogenic HIV-like particle having at least the envelope glycoprotein of a primary isolate of HIV-1 and an attenuated viral vector expressing at least an envelope glycoprotein of a primary isolate of HIV-1.

2. The method of claim 1 wherein said primary isolate is Bx08.

3. The method of claim 2 wherein said DNA molecule is contained in a plasmid vector under the control of a heterologous promoter for expression of the envelope glycoprotein in the host.

4. The method of claim 3 wherein the promoter is the cytomegalovirus promoter.

5. The method of claim 4 wherein the vector has the identifying characteristics of pCMV3Bx08 shown in Figure 2.

6. The method of claim 1 wherein the at least one administration of a priming antigen is at least two administrations of the priming antigen.

7. The method of claim 6 wherein the at least one specific resting period is effected after each priming administration.

8. The method of claim 1 wherein the at least one specific resting period is between about 2 months to about 12 months.

9. The method of claim 1 wherein said non-infectious, non-replicating, immunogenic HIV-like particle comprises an assembly of:

(i) an *env* gene product,

(ii) a *pol* gene product, and

(iii) a *gag* gene product,

said particle being encoded by a modified HIV genome deficient in long terminal repeats (LTRs) and containing gag, pol and env in their natural genomic arrangement.

10. The method of claim 9 wherein the *env* gene is that from primary isolate BX08.

11 The method of claim 1 wherein said non-infectious, non-replicating, immunogenic HIV-like particle is administered in conjunction with an adjuvant.

12. The method of claim 11 wherein the adjuvant is QS21.

13. The method of claim 1 wherein said attenuated viral vector is an attenuated avipoxvirus

14. The method of claim 13 wherein the attenuated viral vector contains a modified HIV-genome deficient in long terminal repeats, wherein at least the *env* gene is that from primary isolate BX08.

15. The method of claim 14 wherein the attenuated avipoxvirus vector is the attenuated canary poxvirus ALVAC.

16. The method of claim 15 wherein the attenuated canary poxvirus vector has the identifying characteristics of vCP1579.

17. The method of claim 1 wherein the at least one administration of a boosting antigen is at least two administrations of a boosting antigen.

18. A vector, comprising a DNA sequence encoding an envelope glycoprotein of a primary isolate of HIV-1 under the control of a heterologous promoter for expression of the envelope glycoprotein in a host organism.

19. The vector of claim 18 wherein the vector is a plasmid vector.

20. The vector of claim 18 wherein said primary HIV-1 isolate is Bx08.

21. The vector of claim 20 wherein the promoter is the cytomegalovirus promoter.

22. The vector of claim 21 which has the identifying characteristics of pCMV3Bx08 shown in Figure 2.

23. The vector of claim 18 wherein the vector is an attenuated viral vector.

24. The vector of claim 23 wherein the attenuated viral vector is a attenuated avipoxvirus vector.

25. The vector of claim 24 wherein the attenuated avipoxvirus vector is the attenuated canary poxvirus vector ALVAC.

26. The vector of claim 25 wherein the attenuated viral vector has the identifying characteristics of vCP1579 shown in Figure 4.

27. A vector, comprising a modified HIV genome deficient in long terminal repeats and a heterologous promoter operatively connected to said genome for expression of said HIV genome in mammalian cells to produce non-infectious, non-replicating and immunogenic HIV-like particles, wherein at least the *env* gene is that from a primary isolate of HIV-1.

28. The vector of claim 27 wherein the vector is a plasmid vector.

29. The vector of claim 28 wherein the primary HIV-1 isolate is BX08.

30. The vector of claim 29 wherein the promoter is type IIA metallothionein promoter.

31. The vector of claim 30 which has the identifying characteristics of p133B1 shown in Figure 3.

Figure 1 Plasmid pCMV.Bx08.gp160

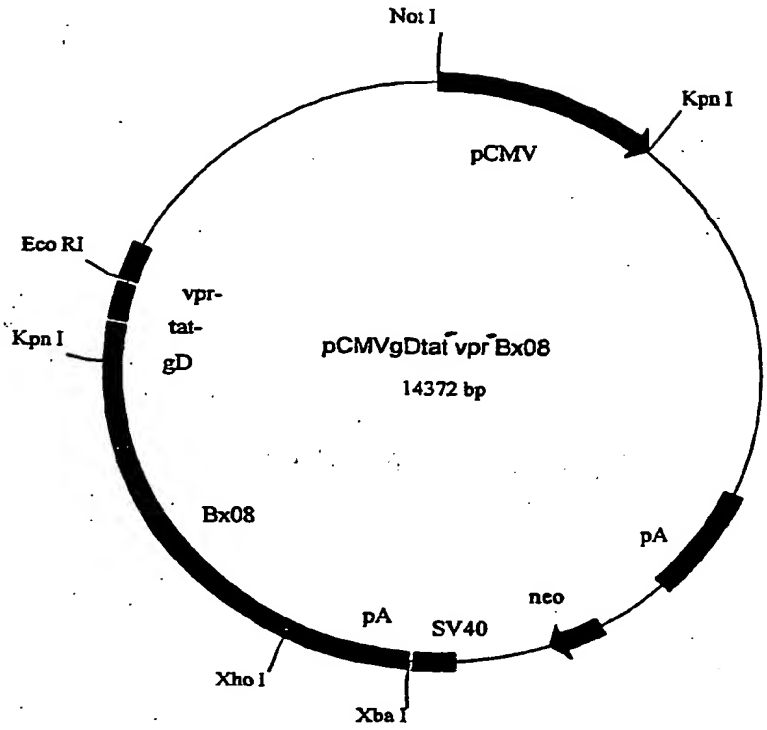


Figure 2 DNA immunization plasmid pCMV3Bx08.

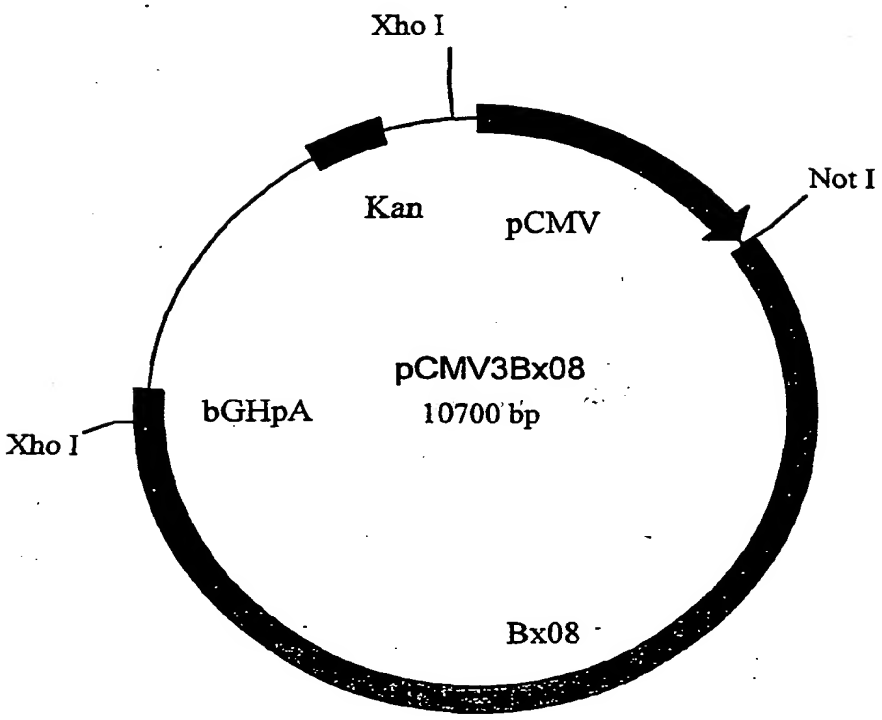


Figure 3. Pseudovirion Expression Plasmid p133B1 HIV-1 Bx08

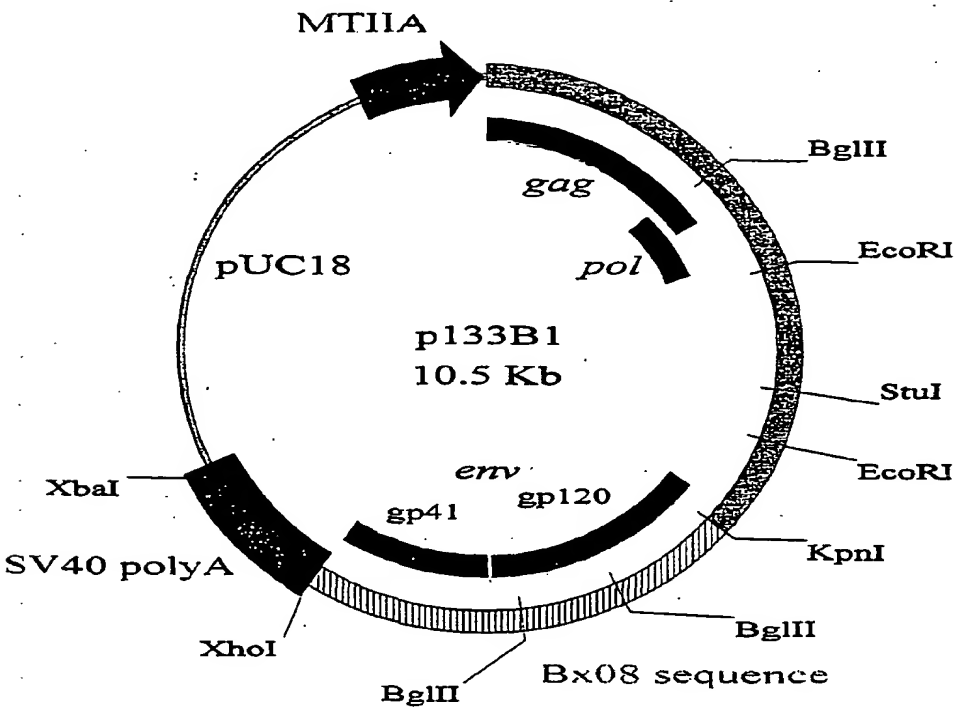
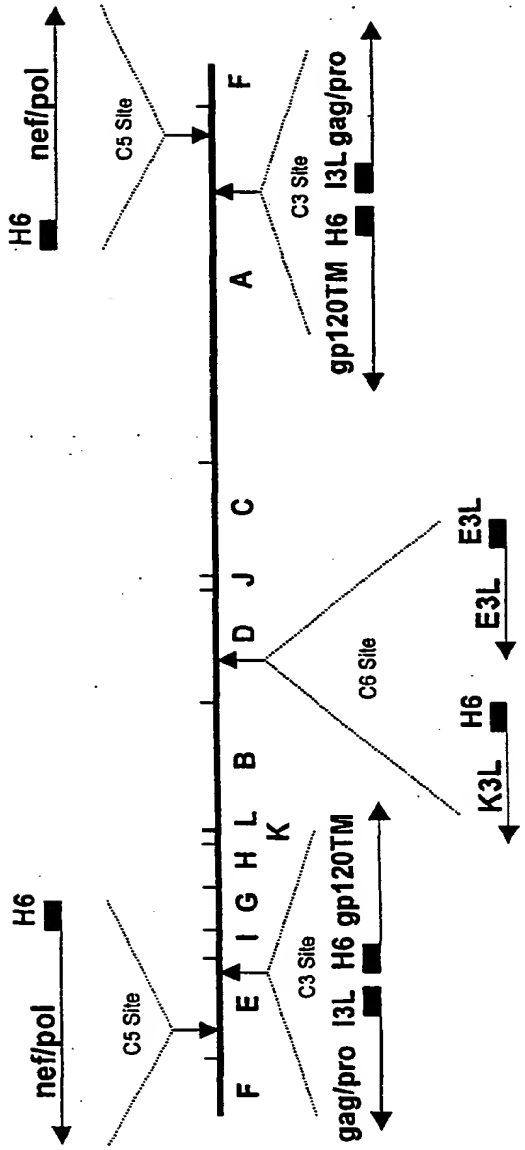


FIGURE 4

ALVAC(2)120(BX08)GNP (vCP1579)

(ALVAC *Xho*I Restriction Map)



A5a

ure 1. Time line of ~~PMC~~ vaccine al

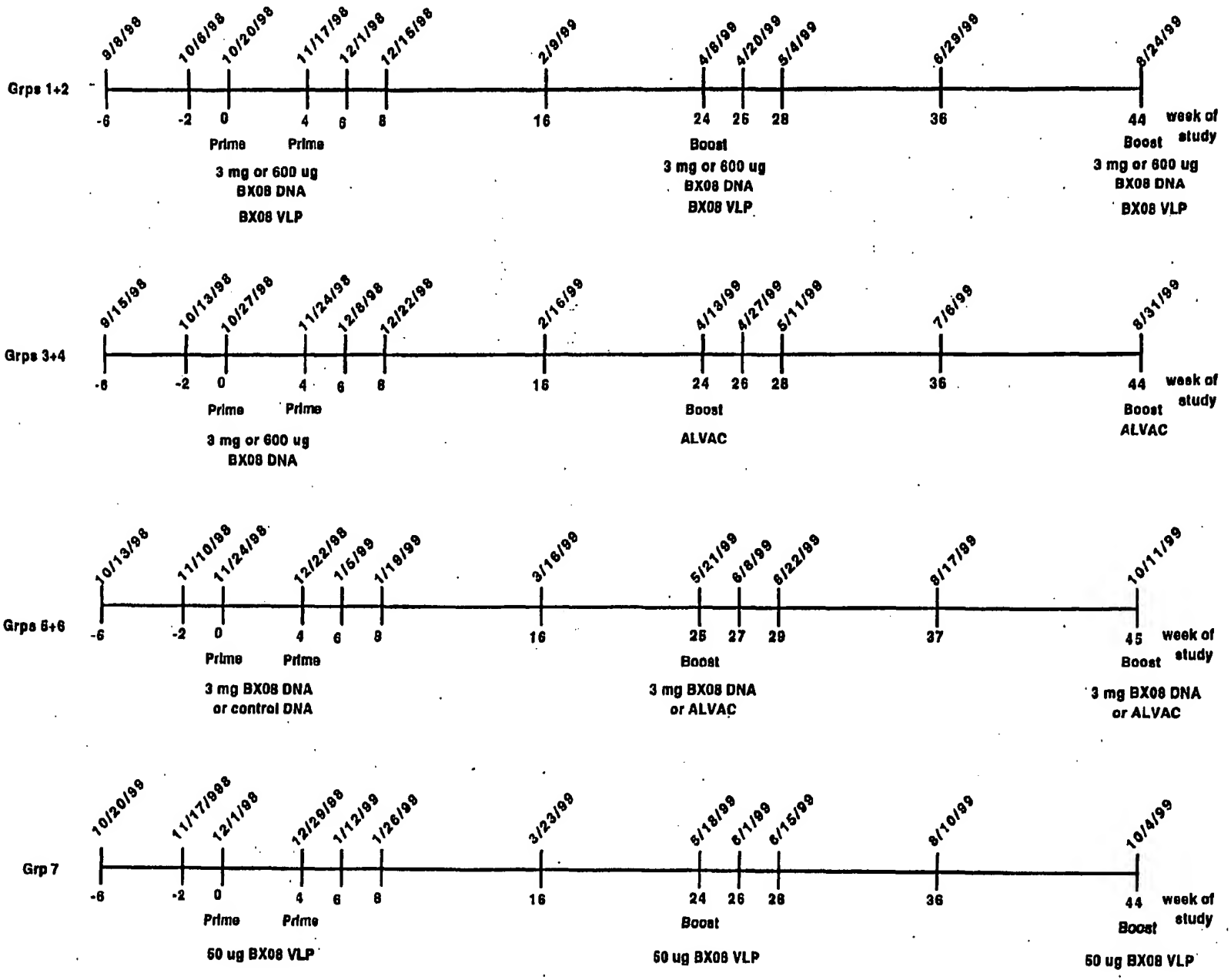
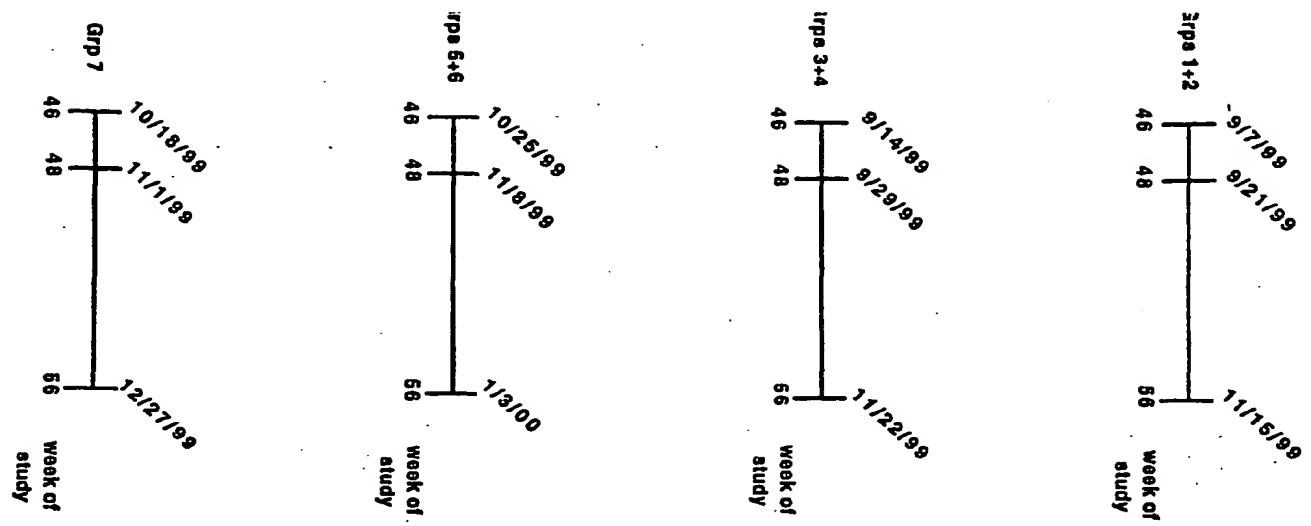


Figure 1 continued



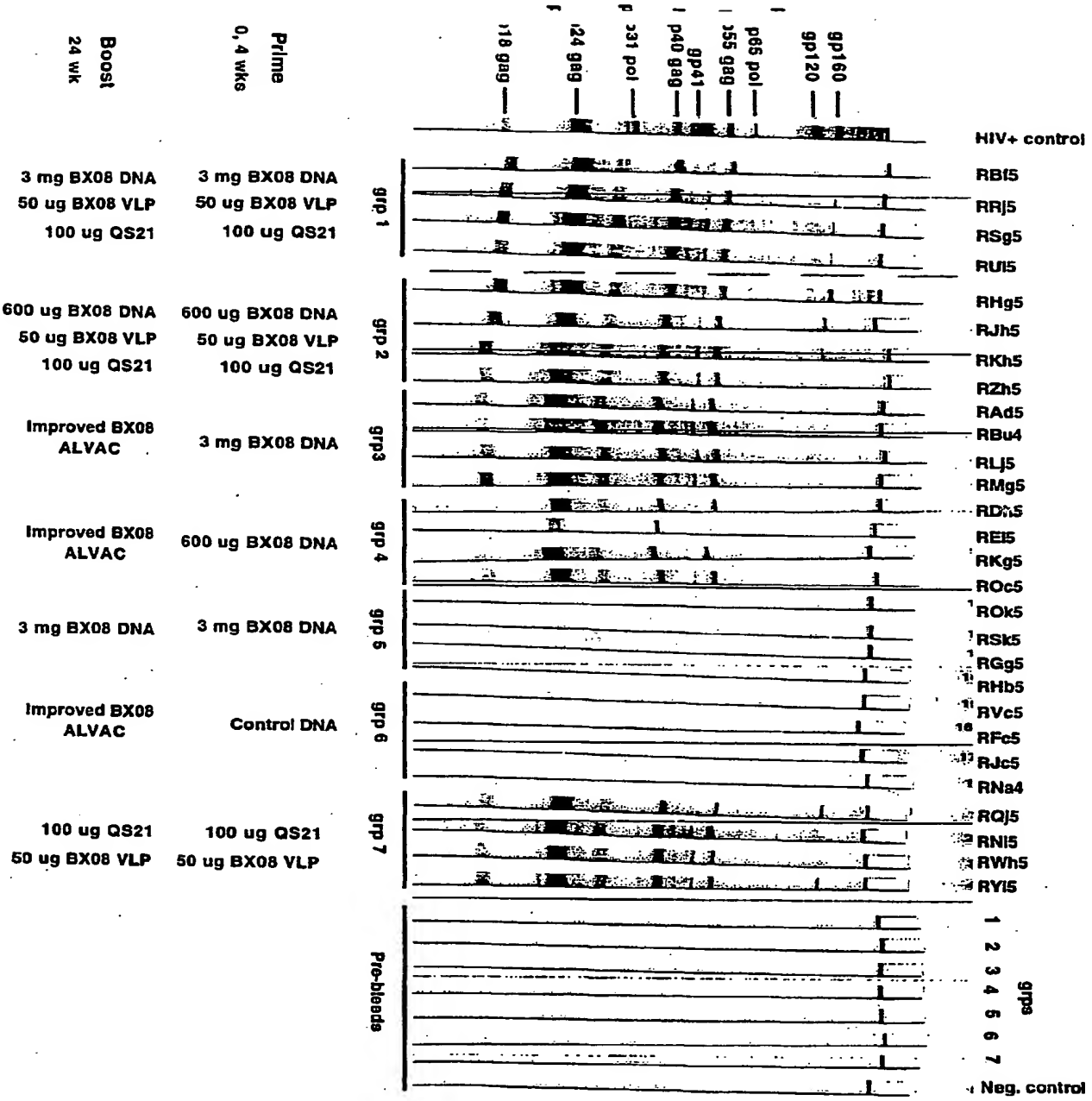


Figure 2. 26 wk macaque um immunoreactivity to HIV an ns (1:100 dln)

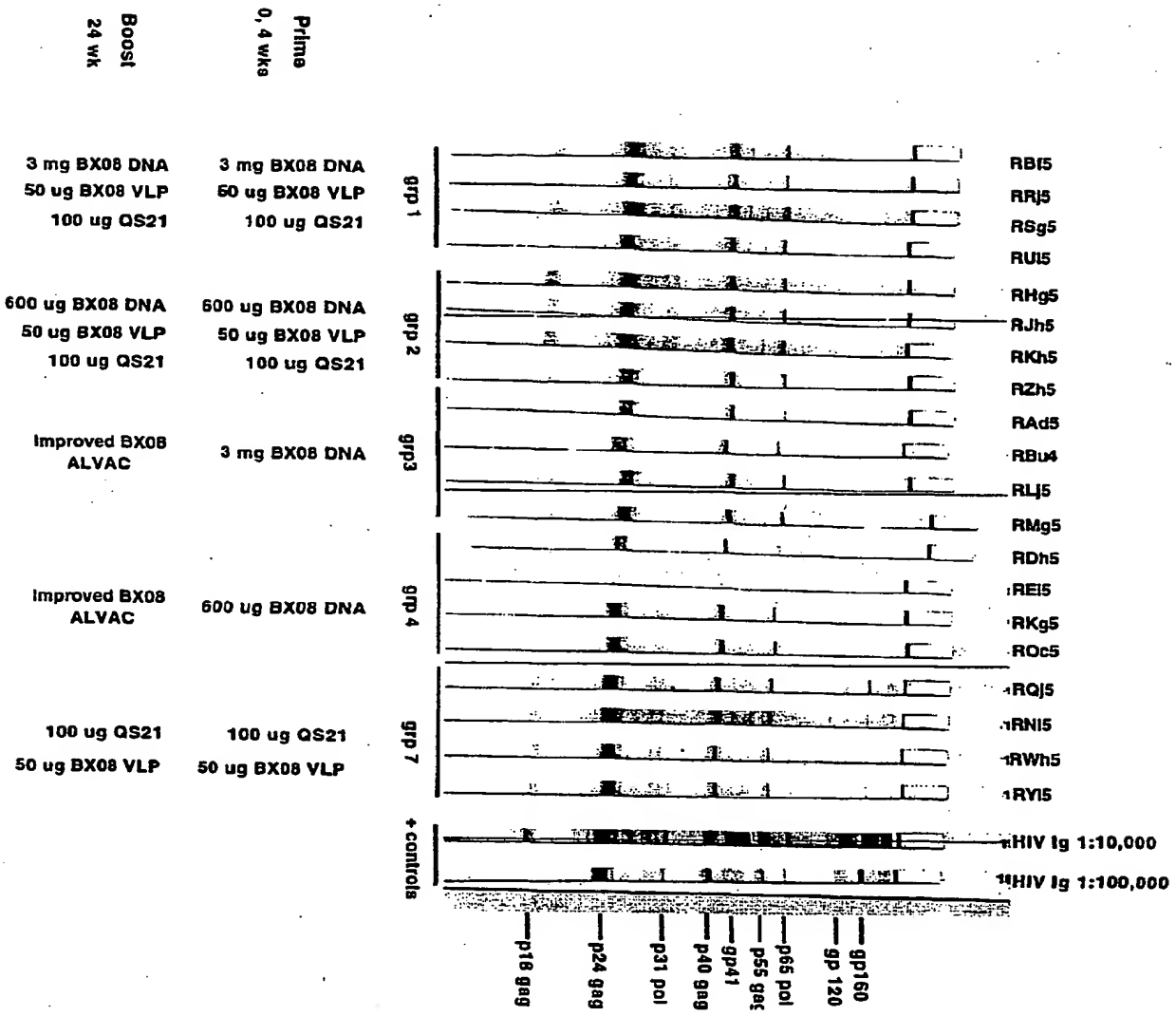
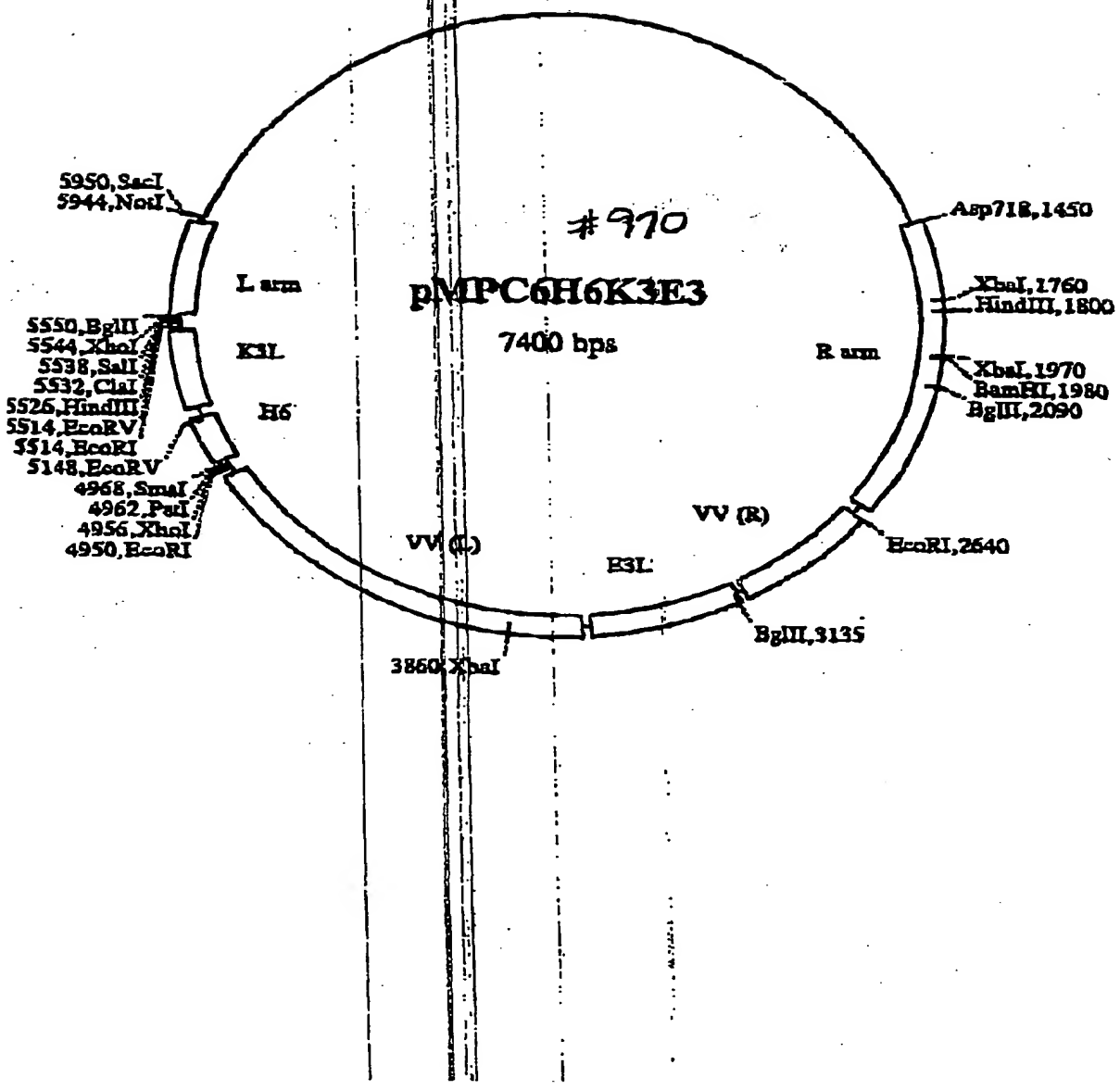


Figure 2: 26 wk macaque serum immunoreactivity to HIV antigens (1:1000 dilln)

647

2-14-POX R

7.8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L arm
K3L
H6
R arm
VV (L)
VV (R)
E3L
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5550,BglII
5544,XhoI
5538,SalI
5532,ClaI
5526,HindIII
5514,EcoRV
5514,EcoRI
5148,EcoRV
4968,SmaI
4962,PstI
4956,XhoI
4950,EcoRI
3860,XbaI
BglII,3135
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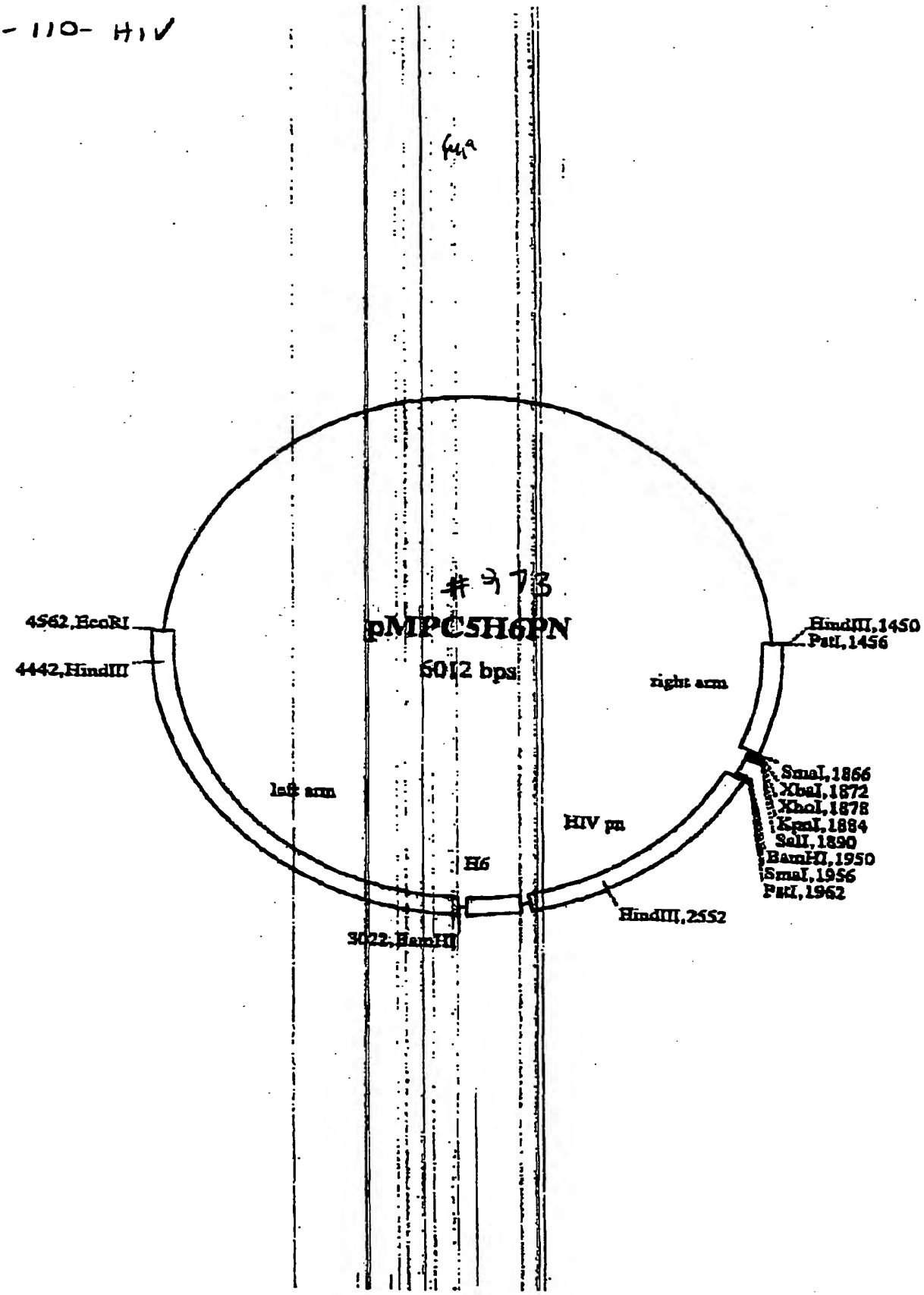
BglII,2090

2-110- HIV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XhoI,1878
KpnI,1884
SalI,1890
BamHI,1950
SmaI,1956
PstI,1962
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HIV pr
H6
HindIII,2552
3022,BamHI

Figure 10 Plasmid pHIV76

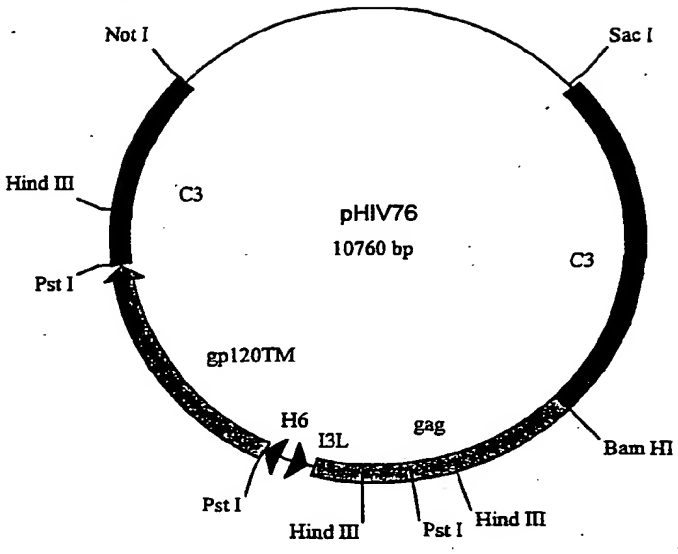


Figure 11

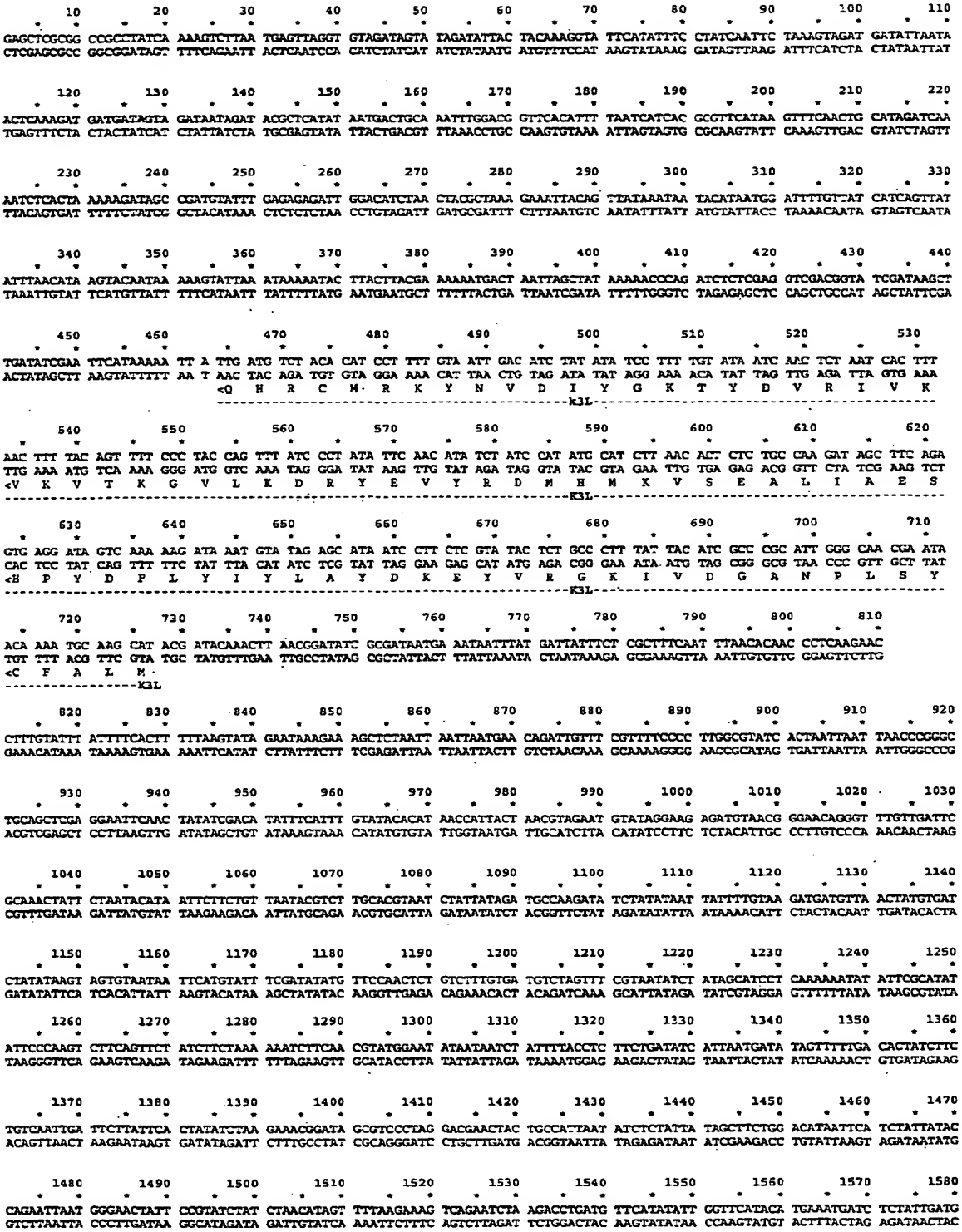
vCP1579: H6/HIV Pol/Nef epitope cassette in ALVAC C5 site

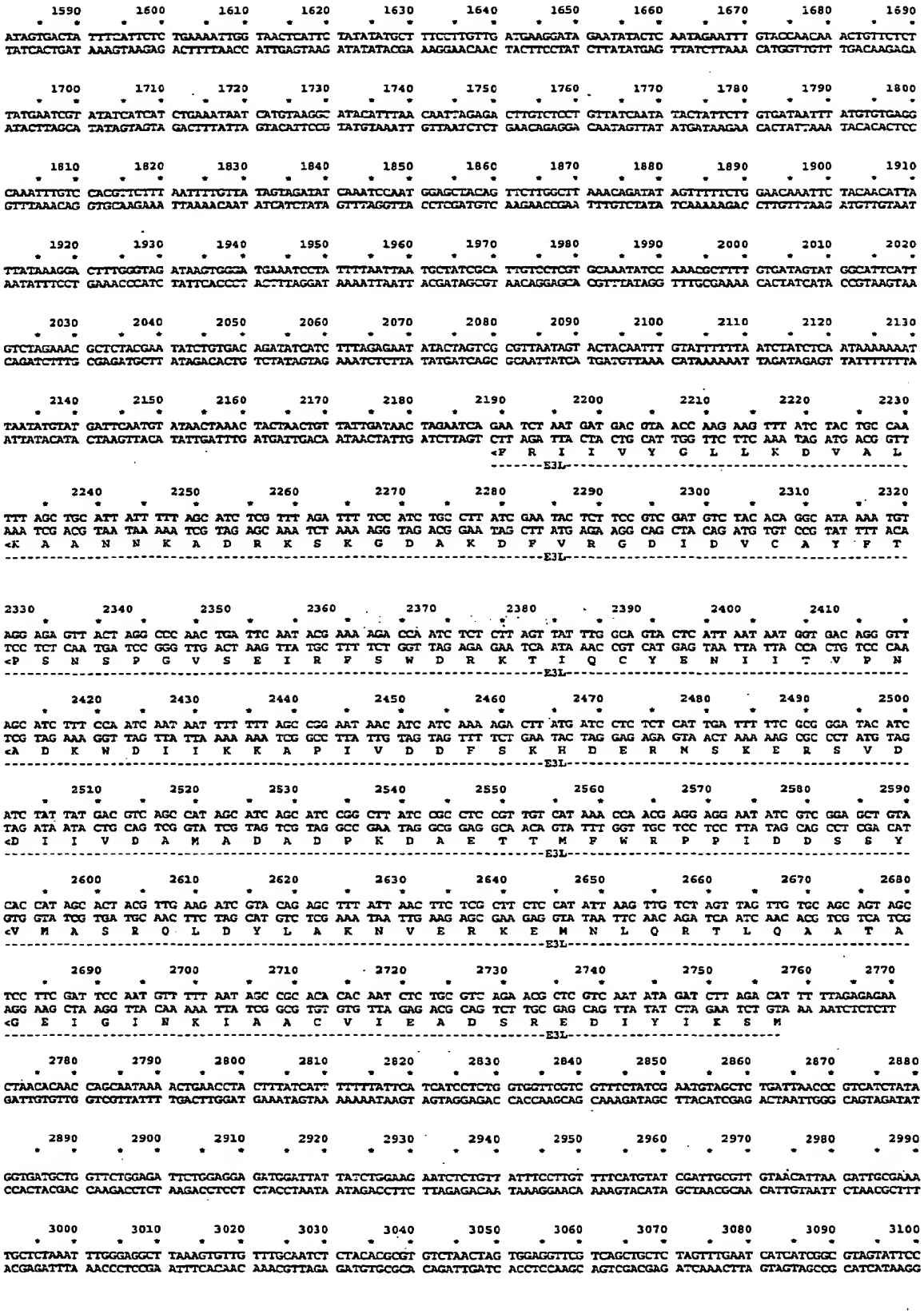
```
   1 TTTTTTTCAT TATTTAGAAA TTATGCATTT TAGATCTTTA TAAGCGGCCG TGATTAACTA
  61 GTCATAAAAA CCCGGGATCG ATTCTAGACT CGAGGGTACC GGATCTTAAT TAATTAGTCA
 121 TCAGGCAGGG CGAGAACGAG ACTATCTGCT CGTTAATTAA TTAGGTCGAC GGATCCCCCA
 181 ACAAAAACTA ATCAGCTATC GGGGTTAATT AATTAGTTAT TAGACAAGGT GAAAACGAAA
 241 CTATTTGTAG CTTAATTAAT TAGAGCTTCT TTATTCTATA CTTAAAAAGT GAAAATAAAT
 301 ACAAAGGTTC TTGAGGGTTG TGTTAAATTG AAAGCGAGAA ATAATCATAA ATTATTTCAT
 361 TATCGCGATA TCCGTTAAGT TTGTATCGTA ATGCCACTAA CAGAAGAAGC AGAGCTAGAA
 421 CTGGCAGAAA ACAGAGAGAT TCTAAAAGAA CCAGTACATG GAGTGTATTA TGACCCATCA
 481 AAAGACTTAA TAGCAGAAAT ACAGAAGCAG GGGCAAGGCC AATGGACATA TCAAATTTAT
 541 CAAGAGCCAT TTAAAAATCT GAAAACAGGA ATGGAGTGGA GATTTGATTC TAGATTAGCA
 601 TTTCATCACG TAGCTAGAGA ATTACATCCT GAATATTTTA AAAATTGTAT GGCAATATTC
 661 CAAAGTAGCA TGACAAAAAT CTTAGAGCCT TTTAGAAAAC AAAATCCAGA CATAGTTATC
 721 TATCAATACA TGGATGATTT GTATGTAGGA TCTGACTTAG AAATAGGGCA GCATAGAACA
 781 AAAATAGAGG AGCTGAGACA ACATCTGTTG AGGTGGGGAC TTACAACCAT GGTAGGTTTT
 841 CCAGTAACAC CTCAAGTACC TTTAAGACCA ATGACTTACA AAGCAGCTGT AGATCTTTCT
 901 CACTTTTTAA AAGAAAAAGG AGGTTTAGAA GGGCTAATTC ATTCTCAACG AAGACAAGAT
 961 ATTCTTGATT TGTGGATTTA TCATACACAA GGATATTTTC CTGATTGGCA GAATTACACA
1021 CCAGGACCAG GAGTCAGATA CCCATTAACC TTTGGTTGGT GCTACAAGCT AGTACCAATG
1081 ATTGAGACTG TACCAGTAAA ATTAAAGCCA GGAATGGATG GCCCAAAAGT TAAACAATGG
1141 CCATTGACAG AAGAAAAAAT AAAAGCATTA GTAGAAATTT GTACAGAGAT GGAAAAGGAA
1201 GGGAAAATTT CAAAAATTGG GCCTTAATTT TTCTGCAGCC CGGGGGATCC TTTTTATAGC
1261 TAATTAGTCA CGTACCTTTG AGAGTACCAC TTCAGCTACC TCTTTTGTGT CTCAGAGTAA
1321 CTTTCTTTAA TCAATTCCAA AACAG
```

Upstream (right) flanking sequence: 1-266
VV H6 promoter: 267-390
HIV pol/nef/pol/nef/pol cassette: 391-1227
Downstream (left) flanking sequence: 1227-1345

Figure 12

E3L and K3L genes in C6





```
     3110       3120       3130       3140       3150       3160       3170       3180       3190       3200       3210
      *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *
TACTTTTACA GTTAGGACAC GGTGTATTGT ATTTCTCGTC GAGAACGTTA AAATAATCGT TGTAACTCAC ATCCTTTATT TTATCTATAT TGTATTCTAC TCCTTTCTTA
ATGAAAATGT CAATCCTGTG CCACATAACA TAAAGAGCAG CTCTTGCAAT TTTATTAGCA ACATTGAGTG TAGGAAATAA AATAGATATA ACATAAGATG AGGAAAGAAT

     3220       3230       3240       3250       3260       3270       3280       3290       3300       3310       3320
      *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *
ATGCATTTTA TACCGAATAA GAGATAGCGA AGGAATTCTT TTTATTGATT AACTAGTCAA ATGAGTATAT ATAATTGAAA AAGTAAAATA TAAATCATAT AATAATGAAA
TACGTAAAAT ATGGCTTATT CTCTATCGCT TCCTTAAGAA AAATAACTAA TTGATCAGTT TACTCATATA TATTAACTTT TTCATTTTAT ATTTAGTATA TTATTACTTT

     3330       3340       3350       3360       3370       3380       3390       3400       3410       3420       3430
      *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *
CGAAATATCA GTAATAGACA GGAACTGGCA GATTCTTCTT CTAATGAAGT AAGTACTGCT AAATCTCCAA AATTAGATAA AAATGATACA GCAAATACAG CTTCATTCAA
GCTTTATAGT CATTATCTGT CCTTGACCGT CTAAGAAGAA GATTACTTCA TTCATGACGA TTTAGAGGTT TTAATCTATT TTTACTATGT CGTTTATGTC GAAGTAAGTT

     3440       3450       3460       3470       3480       3490       3500       3510       3520       3530       3540
      *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *
CGAATTACCT TTTAATTTTT TCAGACACAC CTTATTACAA ACTAACTAAG TCAGATGATG AGAAAGTAAA TATAAATTTA ACTTATGGGT ATAATATAAT AAAGATTCAT
GCTTAATGGA AAATTAAAAA AGTCTGTGTG GAATAATGTT TGATTGATTC AGTCTACTAC TCTTTCATTT ATATTTAAAT TGAATACCCA TATTATATTA TTTCTAAGTA

     3550       3560       3570       3580       3590       3600       3610       3620       3630       3640       3650
      *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *
GATATTAATA ATTTACTTAA CGATGTTAAT AGACTTATTC CATCAACCCC TTCAAACCTT TCTGGATATT ATAAAATACC AGTTAATGAT ATTAAAATAG ATTGTTTAAG
CTATAATTAT TAAATGCATT GCTACAATTA TCTGAATAAG GTAGTTGGGG AAGTTTGGAA AGACCTATAA TATTTTATGG TCAATTACTA TAATTTTATC TAACAAATTC

     3660       3670       3680       3690       3700       3710       3720       3730       3740       3750       3760
      *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *
AGATGTAAAT AATTATTTGG AGGTAAAGGA TATAAAATTA GTCTATCTTT CACATGGAAA TGAATTACCT AATATTAATA ATTATGATAG GAATTTTTTA GGATTTACAG
TCTACATTTA TTAATAAACC TCCATTTCCT ATATTTTAAT CAGATAGAAA GTGTACCTTT ACTTAATGGA TTATAATTAT TAATACTATC CTTAAAAAAT CCTAAATGTC

     3770       3780       3790       3800       3810       3820       3830       3840       3850       3860       3870
      *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *
CTGTTATATG TATCAACAAT ACAGGCAGAT CTATGGTTAT GGTAAAACAC TGTAACGGGA AGCAGCATTC TATGGTAACT GGCCTATGTT TAATAGCCAG ATCATTTTAC
GACAATATAC ATAGTTGTTA TGTCCGTCTA GATACCAATA CCATTTTGTG ACATTGCCCT TCGTCGTAAG ATACCATTGA CCGGATACAA ATTATCGGTC TAGTAAAATG

     3880       3890       3900       3910       3920       3930       3940       3950       3960       3970       3980
      *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *
TCTATAAACA TTTTACCACA AATAATAGGA TCCTCTAGAT ATTTAATATT ATATCTAACA ACAACAAAAA AATTTAACGA TGTATGGCCA GAAGTATTTT CTACTAATAA
AGATATTTGT AAAATGGTGT TTATTATCCT AGGAGATCTA TAAATTATAA TATAGATTGT TGTTGTTTTT TTAAATTGCT ACATACCGGT CTTCATAAAA GATGATTATT

     3990       4000       4010       4020       4030       4040       4050       4060       4070       4080       4090
      *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *
AGATAAAGAT AGTCTATCTT ATCTACAAGA TATGAAAGAA GATAATCATT TAGTAGTAGC TACTAATATG GAAAGAAATG TATACAAAAA CGTGGAAGCT TTTATATTAA
TCTATTTCTA TCAGATAGAA TAGATGTTCT ATACTTTCTT CTATTAGTAA ATCATCATCG ATGATTATAC CTTTCTTTAC ATATGTTTTT GCACCTTCGA AAATATAATT

     4100       4110       4120       4130       4140       4150       4160       4170       4180       4190       4200
      *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *
ATAGCATATT ACTAGAAGAT TTAAAATCTA GACTTAGTAT AACAAAACAG TTAAATGCCA ATATCGATTC TATATTTCAT CATAACAGTA GTACATTAAT CAGTGATATA
TATCGTATAA TGATCTTCTA AATTTTAGAT CTGAATCATA TTGTTTTGTC AATTTACGGT TATAGCTAAG ATATAAAGTA GTATTGTCAT CATGTAATTA GTCACTATAT

     4210       4220       4230       4240       4250       4260       4270       4280       4290       4300       4310
      *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *
CTGAAACGAT CTACAGACTC AACTATGCAA GGAATAAGCA ATATGCCAAT TATGTCTAAT ATTTTAACTT TAGAACTAAA ACGTTCTACC AATACTAAAA ATAGGATACG
GACTTTGCTA GATGTCTGAG TTGATACGTT CCTTATTCGT TATACGGTTA ATACAGATTA TAAAATTGAA ATCTTGATTT TGCAAGATGG TTATGATTTT TATCCTATGC

     4320       4330       4340       4350       4360       4370       4380       4390       4400       4410       4420
      *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *       *  *
TGATAGGCTG TTAAAAGCTG CAATAAATAG TAAGGATGTA GAAGAAATAC TTTGTTCTAT ACCTTCGGAG GAAAGAACTT TAGAACAACT TAAGTTTAAT CAAACTTGTA
ACTATCCGAC AATTTTCGAC GTTATTTATC ATTCCTACAT CTTCTTTATG AAACAAGATA TGGAAGCCTC CTTTCTTGAA ATCTTGTTGA ATTCAAATTA GTTTGAACAT

     4430
      *  *
TTTATGAAGG TACC
AAATACTTCC ATGG
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